# Synthesis and Characterization of Injectable Poly(*N*-isopropylacrylamide)-Based Hydrogels That Support Tissue Formation in Vitro

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ABSTRACT: Hydrogels that support tissue formation in vitro were developed using poly(*N*-isopropylacrylamide) [P(NIPAAm)]. Loosely cross-linked P(NIPAAm) and poly(NIPAAm-*co*-acrylic acid) [P(NIPAAm-*co*-AAc)] hydrogels were synthesized with *N,N′*-methylenebis(acrylamide) cross-linker. At room temperature (RT), the hydrogels were transparent and extremely pliable, while at 37 °C, the matrices became opaque and were significantly more rigid. The P(NIPAAm-*co*-AAc) hydrogel demonstrated significantly less volume change between RT and 37 °C, contained significantly more water at 37 °C, and had a lower critical solution temperature which was significantly higher, as compared to that of the P(NIPAAm) hydrogel. The hydrogels supported bovine articular chondrocyte viability for at least 28 days of in vitro culture, and cartilage-like tissue was formed in the matrices. These hydrogels can be injected through a small-diameter aperture and offer the benefit of in situ stabilization without the possible deleterious effects of in situ polymerization. Thus, these hydrogels have the potential to be used as injectable scaffolds for tissue engineering applications.

## Introduction

Tissues, such as cartilage,[1−3] bone,[4] blood vessels,[5] and peripheral nerves,[6] and organs, such as liver,[7] skin,[8] and pancreas,[9] have been studied extensively in many tissue engineering initiatives. A common approach to repair damaged or diseased tissues and organs employs three-dimensional (3D) polymer matrices that foster regeneration both in vitro and in vivo. Isolated cells are seeded into the polymer matrices, or scaffolds, and the 3D structures guide the cells' organization and development into tissues and organs.[10,11] Ideally, the polymer scaffold should mimic the in vivo environment as accurately as possible and provide a milieu that allows the cells to proliferate, differentiate, maintain their natural phenotype, and ultimately function as a tissue or an organ. Natural polymers, such as alginate,[3] hyaluronic acid,[6] and type I collagen,[2,8] and synthetic polymers, such as poly(glycolic acid),[1,5] poly(lactic acid),[7] and copolymers of poly(glycolic acid) and poly(lactic acid),[4] have been used to synthesize scaffolds for tissue and organ repair. Depending on the ductility of the polymer matrix, the scaffolds can range from being extremely rigid to soft and pliable. Rigid scaffolds must be implanted surgically, while more ductile scaffolds can be injected, reducing the invasiveness of the implantation procedure. In some instances, injectable scaffolds may be more suitable for treating irregularly shaped defects, since rigid scaffolds may be difficult to form into aberrant configurations.

In developing an injectable polymer scaffold for tissue engineering applications, one strategy could be to employ the principles of lower critical phase separation. Polymer mixtures that demonstrate this behavior phase-separate as the temperature is increased above the lower critical solution temperature (LCST).[12,13] Lower critical phase separation is generally regarded as a phenomenon governed by the balance of hydrophilic and hydrophobic moieties on the polymer chain[14] and driven by a negative entropy of mixing.[14−16] In addition, the temperature dependence of certain molecular interactions, such as hydrogen bonding and hydrophobic effects, contribute to this type of phase separation.[17] At the LCST, the hydrogen bonding between the polymer and water becomes unfavorable compared to polymer−polymer and water−water interactions,[16] and an abrupt transition occurs as the hydrated hydrophilic macromolecule quickly dehydrates and changes to a more hydrophobic structure.[16,18]

Poly(*N*-isopropylacrylamide) [P(NIPAAm)] exhibits lower critical phase separation from water at an LCST of approximately 32 °C.[15] Aqueous P(NIPAAm) solutions turn cloudy above the LCST because the hydrophobic groups in the polymer chain form insoluble aggregates.[19] This behavior is reversible, and the P(NIPAAm) chains will dissolve again in water as the temperature is lowered below the LCST, although the kinetics of redissolution are often slower than the precipitation process.[20] P(NIPAAm) hydrogels, formed by polymerizing the hydrophilic NIPAAm monomer with small amounts of cross-linking agents, experience a volume phase transition at the LCST and collapse substantially as the temperature is increased above the LCST.[21,22] During the volume phase transition, the hydrogel expels a large amount of its pore water and generally becomes stiff and opaque.[20,21] Again, this behavior is reversible, and the P(NIPAAm) hydrogel will reswell in water as the temperature is decreased below the LCST, but at a

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rate slower than that of the initial deswelling process.[20] Okano and co-workers have studied the deswelling kinetics of homopolymer and hydrophobically modified P(NIPAAm) hydrogels.[17,23,24] These hydrogels tend to shrink rapidly at first and then deswell slowly, taking more than a month to reach equilibrium deswelling. A "dense skin layer", seen on the surface of the hydrogels above the LCST, is believed to hinder the release of hydrogel water and limit the shrinkage rate.[24]

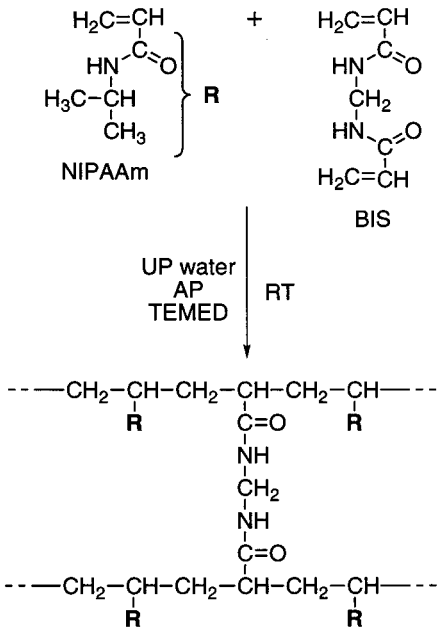
The phase behavior of P(NIPAAm) chains and hydrogels can be manipulated by the addition of more hydrophilic or more hydrophobic monomers. Copolymerization of NIPAAm with a more hydrophilic monomer typically increases the LCST of P(NIPAAm) copolymers[9,14] and copolymer hydrogels,[21,23] while incorporation of a more hydrophobic monomer tends to have the opposite effect.[17,23] Additionally, more hydrophilic monomers decrease both the extent of aggregation experienced by P(NIPAAm) copolymer chains[9] and the extent of temperature-sensitive volume change demonstrated by P(NIPAAm) copolymer hydrogels.[23,25]

P(NIPAAm) homopolymer and copolymer chains and cross-linked hydrogels have been studied for use in a number of diverse applications including solute recovery,[26] solute delivery,[21,25] bioseparations,[28] catalytic reaction control,[29] and chromatography.[31] P(NIPAAm)-based systems have also been examined in combination with cells. For example, Bae et al.[9,32] have used P(NIPAAm) copolymers in the development of a biohybrid artificial pancreas, Shimizu and co-workers[30] have employed P(NIPAAm) copolymers in cell microencapsulation applications, and Yamato et al.[33] have investigated the 3D manipulation of cell sheets that have been cultured on P(NIPAAm) surfaces. The evolution of most of these applications was based on the unique phase behavior of P(NIPAAm) in aqueous media.

In this work, P(NIPAAm) served as a model polymer for examining the use of lower critical phase separation systems in tissue engineering applications. We exploited the phase behavior of P(NIPAAm)-based hydrogels in the development of injectable polymer scaffolds that support tissue formation. Previously, injectable scaffolds have been fabricated using such materials as calcium alginate[3] and Pluronics[34] [i.e., copolymers of poly(ethylene oxide) and poly(propylene oxide)]. Calcium alginate is immunogenic and relatively nondegradable,[35] while Pluronics, which form associative networks as a function of temperature and polymer concentration, lack structural integrity and are relatively unstable.

This report details the synthesis and characterization of loosely cross-linked P(NIPAAm) and P(NIPAAm-*co*-acrylic acid) [P(NIPAAm-*co*-AAc)] hydrogels. These cross-linked hydrogels are extremely pliable and fluidlike at room temperature (RT) but demonstrate a phase transition as the matrix warms from RT to body temperature, yielding more rigid structures. Thus, these hydrogels offer the benefit of in situ stabilization without the potential adverse effects of in situ polymerization. Furthermore, the covalent cross-links give the matrix greater mechanical integrity and physical stability,[36] and the hydrogels can be modified to incorporate biodegradation and biological recognition capabilities. The hydrogels synthesized in this study are injectable, without appreciable macroscopic fracture, through a syringe with a 2 mm diameter aperture and support cartilage-like tissue formation in vitro when seeded with bovine articular chondrocytes.

**Scheme 1**



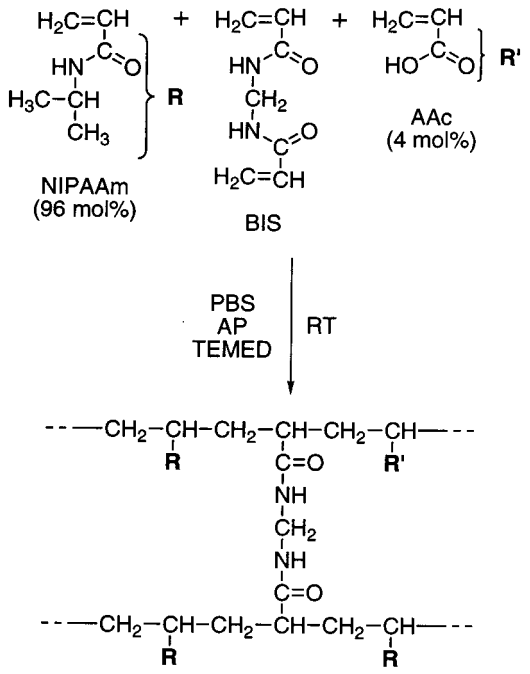

## Experimental Section

**Materials.** *N*-Isopropylacrylamide (NIPAAm), *N*,*N*′-methylenebis(acrylamide) (BIS), Chemzymes Ultrapure grade, ammonium peroxydisulfate (AP), Chemzymes Ultrapure grade, and *N*,*N*,*N*′,*N*′,-tetramethylethylenediamine (TEMED), Chemzymes Ultrapure grade, were obtained from Polysciences, Inc. (Warrington, PA). Acrylic acid (AAc), ethyl alcohol, and deuterium dioxide ($D_2O$) were purchased from Aldrich (Milwaukee, WI). Pronase protease was obtained from Calbiochem-Novabiochem Corp. (LaJolla, CA). Collagenase P was purchased from Boehringer Mannheim (Mannheim, Germany). Dimethyl sulfoxide (DMSO) and L-ascorbic acid were obtained from Sigma (St. Louis, MO). Dulbecco's modified eagle medium [(DMEM), high glucose, with L-glutamine, with pyridoxine hydrochloride, without sodium pyruvate], Hanks' balanced salt solution [(HBSS), without calcium chloride, without magnesium chloride, without magnesium sulfate, without phenol red], Dulbecco's phosphate-buffered saline [(PBS), without calcium chloride, without magnesium chloride; pH = 7.2 ± 0.1], penicillin−streptomycin (P/S), trypsin−ethylenediamine tetraacetic acid (trypsin/EDTA), and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). Fluorescein diacetate (F-DA) was obtained from Molecular Probes (Eugene, OR). All materials were used as received.

**Synthesis of the Injectable P(NIPAAm) Hydrogel.** Dry nitrogen gas was bubbled through a mixture of 2.5 g (0.0221 mol) of NIPAAm, 0.0027−0.0040 g (0.0175−0.0260 mmol) of BIS, and 50 mL of ultrapure (UP) water (18 MΩ-cm; pH = 7.2 ± 0.1) in a three-neck distilling flask for 15 min to remove dissolved oxygen. Following the nitrogen gas purge, 0.020 g (0.0876 mmol) of AP and 200 μL (0.0013 mol) of TEMED were added as the initiator and the accelerator, respectively. The mixture was stirred vigorously for 15 s and allowed to polymerize at RT (≈22 °C) for 19 h in a 250 mL glass beaker covered with a glass plate. Following polymerization, the P(NIPAAm) hydrogel was washed three times, 15−20 min each, in UP water to extract unreacted compounds. A schematic representation of the P(NIPAAm) hydrogel synthesis is shown in Scheme 1.

**Synthesis of the Injectable P(NIPAAm-*co*-AAc) Hydrogel.** Dry nitrogen gas was bubbled through a mixture of 2.435 g (0.0215 mol) of NIPAAm, 0.065 g (0.903 mmol) of AAc, 0.004−0.005 g (0.0260−0.0325 mmol) of BIS, and 50 mL of PBS in a three-neck distilling flask for 15 min. Following the nitrogen gas purge, 0.020 g of AP (0.0876 mmol) and 200 μL (0.0013 mol) of TEMED were added as the initiator and accelerator, respectively. The mixture was stirred vigorously

**Scheme 2**




for 15 s and allowed to polymerize at RT (≈22 °C) for 19 h in a 250 mL glass beaker covered with a glass plate. Following polymerization, the P(NIPAAm-*co*-AAc) hydrogel was washed three times, 15−20 min each, in UP water to extract unreacted compounds. A schematic representation of the P(NIPAAm-*co*-AAc) hydrogel synthesis is presented in Scheme 2.

**Nuclear Magnetic Resonance (NMR) Spectroscopic Studies.** P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogel samples were dried in a vacuum desiccator for 9 days to remove the hydrogel water. Once dried, the samples were immersed in 50 mL of $D_2O$ and allowed to swell for 4 days. This process was repeated a second time to ensure sufficient replacement of the hydrogel water with $D_2O$ and adequate conversion of the −NH and −OH groups to −ND and −OD groups, respectively.[37,38] The $D_2O$-swollen hydrogel samples were sent to Spectral Data Services, Inc. (Champaign, IL) for solid-state $^1H$ magic angle spinning (MAS) NMR spectroscopy. The spectra were obtained on a 270 MHz spectrometer, and spin speeds of 1.8 and 2.0 kHz were used to analyze the P(NIPAAm) and P(NIPAAm-*co*-AAc) samples, respectively.

**LCST Determination.** P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were immersed in excess PBS for 0, 3, or 6 days at RT to determine the effects of swelling on the LCST. A Beckman DU-64 UV−vis spectrophotometer with a DU series 60 water-regulated single-cell holder (Fullerton, CA) was used to determine the LCST of the hydrogel samples. The transmittance of visible light ($\lambda$ = 500 nm; path length = 1 cm) through the hydrogel was recorded as the hydrogel temperature was varied with a Fisher model 90 refrigerated bath (Pittsburgh, PA). A K-type thermocouple attached to an Omega HH-81 digital thermometer (Stamford, CT) was used to measure the hydrogel temperature. The heating rate was 0.1−0.25 °C/min. At the start of each experiment, the spectrophotometer was calibrated with UP water. Once a plot of transmittance vs temperature was obtained, the LCST was judged to be the initial break point of the curve.[39]

**Water Content Determination of Hydrogels at RT.** P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were immersed in excess PBS for 0, 3, or 6 days at RT to determine the effects of swelling on RT water content. A syringe was used to transfer 5.0 mL samples of hydrogel into preweighed cylindrical copper molds (internal diameter = 26 mm), and the sample weight was determined. The samples were quenched in liquid nitrogen (≈−196 °C) and freeze-dried (VirTis, Gardiner, NY) at a pressure of 30 mTorr. During the freeze-drying process, the samples were allowed to warm from −106 °C to RT over a period of 24 h. The freeze-dried hydrogels were weighed upon removal from the freeze-dryer, and an estimate of the water content was calculated on the basis of the weight difference of the samples before and after freeze-drying. Using the weight difference and the density of water at 22 °C, the volume percent of water in the hydrogel at RT was determined.

**Water Content Determination of Hydrogels at 37 °C.** P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were immersed in excess PBS for 0, 3, or 6 days at RT to determine the effects of swelling on 37 °C water content. A syringe was used to transfer 5.0 mL of a P(NIPAAm) hydrogel into a Corning 35 mm tissue-culture polystyrene dish (Corning, NY). Similarly, 5.0 mL of a P(NIPAAm-*co*-AAc) hydrogel was injected into a preweighed glass vial (internal diameter = 23 mm; volume = 25 mL) containing 3 mL of PBS. In preliminary studies, P(NIPAAm-*co*-AAc) hydrogels collapsed more when heated to 37 °C after being exposed to PBS, while P(NIPAAm) hydrogels collapsed to the same extent whether in the presence of PBS or not. Thus, PBS was added to the glass vials containing the P(NIPAAm-*co*-AAc) hydrogels. The polystyrene dishes and glass vials were placed in a NuAire TS Autoflow $CO_2$ water-jacketed incubator (Plymouth, MN) at a temperature of 37 °C and a humidified atmosphere of 5% $CO_2$ (95% air; 21% $O_2$) for 3 days. The weight of the heated hydrogel samples was determined, and the volume of each heated sample was estimated by the displacement of 37 °C UP water. The time course of the test was short enough to neglect substantial swelling of the hydrogels in the water. In addition, P(NIPAAm)-based hydrogels typically expel water at 37 °C, so significant swelling at 37 °C was not anticipated. The P(NIPAAm) hydrogel samples were transferred to copper molds (internal diameter = 26 mm) that had been preheated to 37 °C. The molds containing the P(NIPAAm) samples and the glass vials containing the P(NIPAAm-*co*-AAc) samples were quenched in liquid nitrogen (≈−196 °C). The samples were freeze-dried for 24 h and weighed at the completion of the freeze-drying process. An estimate of the water content in the heated P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels was calculated on the basis of the weight difference of the samples before and after freeze-drying. Using the weight difference and the density of water at 37 °C, the volume percent of water in the hydrogel at 37 °C was determined.

**Change in Volume Estimation between RT and 37 °C.** P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were immersed in PBS for 0, 3, or 6 days at RT to determine the effects of swelling on volume change. Hydrogel samples (5.0 mL) were heated from RT to 37 °C for 6 days in an incubator at a humidified atmosphere of 5% $CO_2$. The volume of the heated hydrogel samples was determined via 37 °C water displacement. The change in volume between RT and 37 °C for the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels was computed by subtracting the RT volume from the 37 °C volume and dividing by the RT volume.

**Rheology of the P(NIPAAm-*co*-AAc) Hydrogel at RT and 37 °C.** Forceps were used to carefully grasp a small sample from the top of a P(NIPAAm-*co*-AAc) hydrogel (i.e., the side of the hydrogel that did not polymerize in contact with the glass beaker), and scissors were used to cut the sample from the bulk hydrogel. In addition, a 3 mL syringe was used to collect a small sample (≈0.5 mL) from the top of the hydrogel. These samples obtained with scissors (i.e., "cut") and a syringe (i.e., "injected") were tested on a Bohlin VOR rheometer (Bohlin Rheologi, Cranbury, NJ) using a 30 mm diameter parallel plate configuration in oscillatory mode. Sample thickness ranged from 0.648 to 1.241 mm, depending on the hydrogel sample volume. Prior to sample loading, a plastic collar was placed around the lower plate, creating a reservoir. Once the sample was loaded and the upper fixture was lowered, silicone fluid was placed in the reservoir to prevent sample dehydration during data collection. Each sample was first tested at 22 °C and then at 37 °C. The temperature of the lower plate was maintained with a recirculating water bath. Linear viscoelastic data were collected using a 3.61 g cm torsion bar over a frequency range of 0.001−10 Hz. Depending on the sample thickness, the shear strain

ranged from 17.4% to 34.1%. Preliminary experiments demonstrated that the rheological properties were independent of the applied strain in this range and were unaffected by the silicone fluid (data not shown).

**Isolation of Bovine Articular Chondrocytes for in Vitro Studies.** The method used to isolate chondrocytes from bovine articular cartilage was adapted from a previously developed procedure.[40] Briefly, cartilage slices obtained from adult bovine (18−20 months old) metacarpophalangeal joints were digested first in Pronase solution [0.2% (w/v) Pronase in DMEM with 5% FBS (v/v)] for 90 min at 37 °C and then in collagenase solution [0.025% (w/v) collagenase in DMEM with 5% FBS (v/v)] for 16−19 h at 37 °C. The cell pellet obtained from the digestion solution was suspended in HBSS, passed through a 70 $\mu$m nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ), and repeatedly washed in HBSS and centrifuged. The cells were then suspended in DMEM, filtered through a 40 $\mu$m nylon cell strainer (Becton Dickinson and Co.), and placed in media containing DMEM with 10% FBS (v/v), 1% P/S (v/v), and 0.045 mg/mL L-ascorbic acid. The chondrocytes remained in culture overnight in an incubator at a temperature of 37 °C and a humidified atmosphere of 5% $CO_2$ and were added to the injectable hydrogels the day after isolation.

**Seeding Chondrocytes into the Injectable P(NIPAAm) and P(NIPAAm-*co*-AAc) Hydrogels.** P(NIPAAm) hydrogel disks (≈10 mm in diameter; ≈4 mm in height) were formed by heating 5.0 mL samples to 37 °C in an incubator for 3 days. The disks were sterilized in a 70% solution (v/v) of ethyl alcohol in water at RT for 15 min and immersed in sterile water at RT for 4 h. P(NIPAAm-*co*-AAc) hydrogel samples were sterilized in a 70% solution (v/v) of ethyl alcohol in water at RT for 15 min, after which they were soaked in sterile water at RT for 1 h. The P(NIPAAm) disks were placed in the wells of a 12-well dish, and a 5 mL syringe was used to transfer 1.0 mL of P(NIPAAm-*co*-AAc) hydrogel samples into the wells of a 24-well dish. Bovine articular chondrocytes isolated the previous day were released from the tissue-culture flasks by the addition of trypsin/EDTA. The cells were collected by centrifugation, suspended in DMEM, and counted with a hemocytometer. The cell suspension was added to a syringe, and equal volumes (≈1 mL) were injected at RT into the P(NIPAAm) hydrogel disks and the P(NIPAAm-*co*-AAc) hydrogel samples. The initial cell seeding density was approximately $9.6 \times 10^6$ cells/mL of the P(NIPAAm) hydrogel and approximately $2.2 \times 10^6$ cells/mL of the P(NIPAAm-*co*-AAc) hydrogel. The cells were injected throughout the hydrogel sample as evenly as possible. This injection seeding method generated a relatively uniform distribution of cells through the entire scaffold. Following the cell seeding, media [DMEM with 10% FBS (v/v), 1% P/S (v/v), and 0.045 mg/mL L-ascorbic acid] was added to each well, and the dishes were placed in an incubator at a temperature of 37 °C and a humidified atmosphere of 5% $CO_2$ (95% air; 21% $O_2$). The following day, the chondrocyte-loaded hydrogels were transferred to new 12-well dishes. The media was changed every other day during the course of the study.

**In Situ Fluorescent Viability Study.** To assess chondrocyte viability in the hydrogels, an in situ fluorescent viability study with fluorescein diacetate (F-DA) was performed. F-DA, which is not fluorescent, is converted to fluorescein (excitation/emission maxima ≈ 494/520 nm) through the cleavage of its acetate groups by intracellular esterases inside healthy cells. At 13, 21, and 28 days of in vitro culture, one of each of the chondrocyte-loaded P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels was studied. A 60 mM solution of F-DA in DMSO was prepared and diluted to 60 $\mu$M in PBS. The hydrogel sample was immersed in the diluted F-DA solution for 10 min at RT and then washed in PBS for 5 min. Following immersion in F-DA, live chondrocytes in the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were viewed on a Nikon Diaphot inverted microscope (Nippon Kogaku K.K., Tokyo, Japan) using epi-illumination, a Nikon 20× phase objective, and standard Nikon filter sets for fluorescein (B-2A). Fluorescent images were obtained with a Photometrics Ltd. cooled charged coupled device (CCD) camera (Tucson, AZ). Control P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels without any cells were analyzed to determine background fluorescence. Image analysis was performed using Oncor Image (Gaithersburg, MD), Adobe Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA), Canvas 3.5.3 (Deneba Software, Miami, FL), and N.I.H. Image.

Chondrocytes cultured in vitro in alginate beads for 28 days served as the positive control for cell viability and cell shape, while chondrocytes cultured in vitro on the bottom of a tissue-culture dish (i.e., a two-dimensional (2D) surface) for 28 days served as the negative control. The alginate-seeding method was the same as that described by Häuselmann et al.[41] The chondrocyte-loaded alginate beads were placed in media and cultured in vitro for 28 days.

**Histological Analyses.** A chondrocyte-seeded P(NIPAAm) hydrogel cultured in vitro for 90 days and a cell-loaded P(NIPAAm-*co*-AAc) hydrogel cultured in vitro for 36 days were immersed in 10% buffered formalin (Baxter, Deerfield, IL) for 24 h at 37 °C to fix the cells. The P(NIPAAm) sample was embedded in paraffin, sectioned longitudinally, and stained with hematoxylin and eosin (H&E) to study the tissue structure and Alcian blue (pH = 1.0) to demonstrate the presence of sulfated polysaccharides.[42] The P(NIPAAm-*co*-AAc) hydrogel was quenched in liquid nitrogen (≈−196 °C), the frozen sample was sectioned longitudinally with a Reichert-Jung 2800 Frigocut N cryotome (Leica, Zurich, Switzerland), and the sections were stained with H&E and Alcian blue (pH = 1.0). A control P(NIPAAm-*co*-AAc) hydrogel without cells was also stained with H&E and Alcian blue. It should be noted that the hydrogels were tested at different time points due to difficulties encountered during the dehydration and paraffin embedding of the P(NIPAAm) hydrogel.

**Statistical Analyses.** Analysis of variance (ANOVA) tests and Newman-Keuls post-hoc analyses were performed using StatSoft Statistica 5.0 (Tulsa, OK). Statistical significance was determined at a value of $p < 0.01$.

## Results and Discussion

**Synthesis of the P(NIPAAm) and P(NIPAAm-*co*-AAc) Hydrogels.** The loosely cross-linked P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels synthesized in this study were readily injectable through a syringe with a 2 mm diameter aperture and did not exhibit appreciable macroscopic fracture following injection. At RT, the hydrogels were colorless, transparent, and extremely pliable. When heated to 37 °C, the P(NIPAAm) hydrogel exhibited a considerable amount of collapse, released a large fraction of pore water, and became stiff and opaque. The P(NIPAAm-*co*-AAc) hydrogel also became less pliable than at RT and turned cloudy at 37 °C. However, this hydrogel experienced less volume change, released less pore water, and maintained its shape better, as compared to the case of the P(NIPAAm) hydrogel.

**$^1$H NMR Spectroscopic Studies.** Solid-state $^1$H MAS NMR spectra of the $D_2O$-swollen P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels are shown in Figure 1. The P(NIPAAm) hydrogel spectrum shown in Figure 1a contains nine peaks. Peak 1 at 0.915 ppm represents the $-CH_3$ groups in NIPAAm, peak 2 at 1.359 ppm represents the $-CH_2$ groups in the polymer backbone, peak 3 at 1.780 ppm represents the −CH groups in the polymer backbone, and peak 6 at 3.658 ppm represents the isopropyl −CH group in NIPAAm. Theoretically, the intensities of peaks 1, 2, 3, and 6 should be in a ratio of roughly 6:2:1:1, respectively. The measured intensities are in a ratio of 5.6:1.8:0.98:1. These NMR data are consistent with both the chemical structure of the P(NIPAAm) hydrogel and other published reports.[38] Peak 7 at 4.556 ppm represents residual protons in the $D_2O$ used to replace the hydrogel water. Peaks 8 and 9 at 5.477 and 5.948 ppm, respectively, may represent the

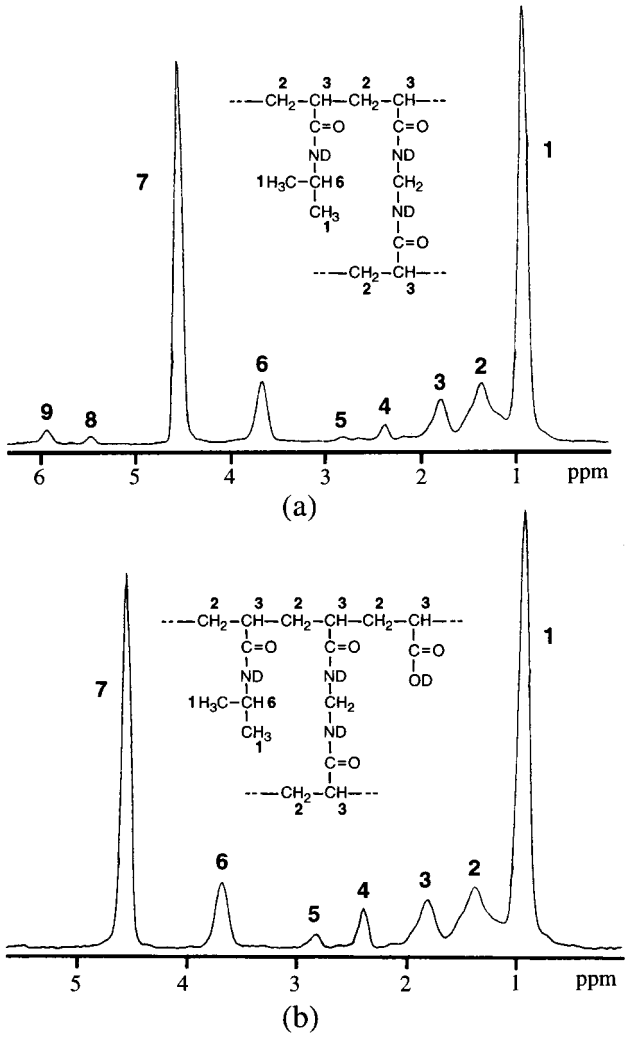



**Figure 1.** Solid-state $^1$H MAS NMR spectra of the (a) P(NIPAAm) and (b) P(NIPAAm-*co*-AAc) hydrogels. The peak numbers correspond to the hydrogen marked on the structural schematic. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

vinyl protons in NIPAAm monomer since the NIPAAm vinyl protons absorb at chemical shifts similar to these peaks (data not shown). This suggests that the P(NIPAAm) hydrogel may not have been washed thoroughly enough following polymerization to remove all of the unreacted NIPAAm monomer.

The P(NIPAAm-*co*-AAc) hydrogel spectrum shown in Figure 1b contains seven peaks. Peak 1 at 0.921 ppm represents the $-CH_3$ protons in NIPAAm, peak 2 at 1.350 ppm represents the $-CH_2$ protons in the polymer backbone, peak 3 at 1.781 ppm represents the $-CH$ proton in the polymer backbone, and peak 6 at 3.658 ppm represents the isopropyl $-CH$ group in NIPAAm. The theoretical intensities of peaks 1, 2, 3, and 6 should be in a ratio of roughly 6:2:1:1, respectively. The measured intensities are in a ratio of 6.2:1.9:1.1:1. These NMR results are consistent with the chemical structure of the P(NIPAAm-*co*-AAc) hydrogel. Peak 7 at 4.547 ppm represents residual protons in the $D_2O$ used to replace the hydrogel water. Importantly, the two peaks in the P(NIPAAm) hydrogel spectrum believed to represent NIPAAm vinyl protons are not present in the P(NIPAAm-*co*-AAc) hydrogel spectrum. This suggests that the P(NIPAAm-*co*-AAc) hydrogel was washed thoroughly enough to remove residual monomer.

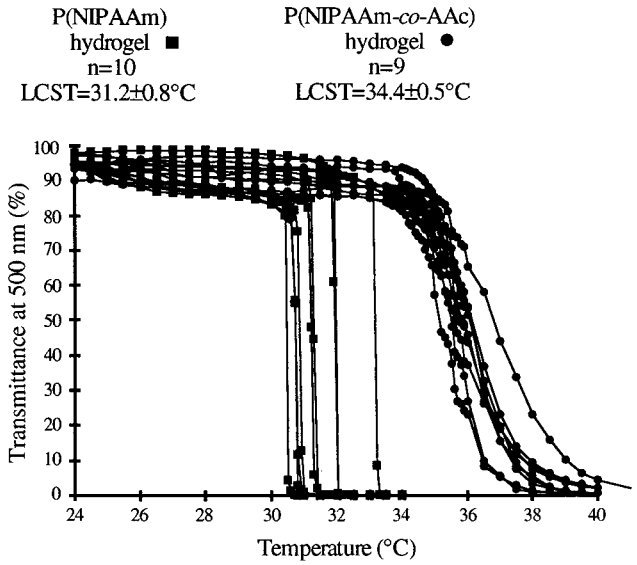



**Figure 2.** Transmittance vs temperature data for the P(NIPAAm) (■) and P(NIPAAm-*co*-AAc) (●) hydrogels. Each line represents a separate experiment. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

Peak 4 at 2.4 ppm and peak 5 at 2.8 ppm in the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogel spectra have not yet been assigned. These small peaks were not present in the NIPAAm monomer, AAc monomer, or BIS spectra (data not shown); however, they were seen in the P(NIPAAm) and P(NIPAAm-*co*-AAc) spectra (data not shown) and may represent isomers and/or end groups of polymer chains formed by different termination steps (e.g., disproportionation vs coupling).

An absorption for the $-CH_2$ protons in the BIS crosslinker was not observed in the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogel spectra. In the BIS spectrum, the $-CH_2$ protons absorbed at 4.5 ppm (data not shown), so the broad $D_2O$ peak in the hydrogel spectra may have covered the peak for the BIS protons. Alternatively, since only 0.0054−0.0080% (w/v) BIS was used to synthesize the P(NIPAAm) hydrogel and 0.008−0.01% (w/v) BIS was used to synthesize the P(NIPAAm-*co*-AAc) hydrogel, the amount of BIS present in the hydrogels may have been too low to be detected.

**LCST Determination.** The effect of temperature on the transmittance of visible light ($\lambda = 500$ nm) through the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels is shown in Figure 2. Each line represents a single experiment with one hydrogel sample. The P(NIPAAm) hydrogel phase transition was very sharp, spanning approximately 0.4 °C, while the P(NIPAAm-*co*-AAc) hydrogel phase transition was much broader and extended roughly 5 °C. The P(NIPAAm) hydrogel LCST was determined to be 31.2 ± 0.8 °C, and the P(NIPAAm-*co*-AAc) hydrogel LCST was found to be 34.4 ± 0.5 °C ($p < 0.01$). The LCSTs were not significantly affected by swelling the hydrogels in PBS for up to 6 days. The LCST of the P(NIPAAm) hydrogel is in agreement with published reports.[16−18,21,26] Furthermore, the extremely sharp transition observed is consistent with results published previously and has been attributed to the hydrophobic/hydrophilic balance of the side groups on the polymer chain which leads to rapid dehydration of the polymer as the temperature is increased above the LCST.[16,18]

The increased LCST of the P(NIPAAm-*co*-AAc) hydrogel also agrees with previous studies.[21,23] In general,

**Table 1. Water Content of the P(NIPAAm) andP(NIPAAm-*co*-AAc) Hydrogels at RT and 37 °C**

| hydrogel | swelling in PBS (days) | RT water content (%) | 37 °C water content (%) |
|---|---|---|---|
| P(NIPAAm) | 0 | 91.5 ± 0.8[a] | 43.7 ± 7.7[a] |
| P(NIPAAm) | 3 | 93.6 ± 0.6[b] | 56.4 ± 9.1[a] |
| P(NIPAAm) | 6 | 95.3 ± 0.8[a] | 67.8 ± 11.9[a] |
| P(NIPAAm-*co*-AAc)[d] | 0 | 92.6 ± 0.7[a] | 93.3 ± 5.4[c] |
| P(NIPAAm-*co*-AAc)[d] | 3 | 93.5 ± 0.9[a] | 82.4 ± 7.1[a] |
| P(NIPAAm-*co*-AAc)[d] | 6 | 92.6 ± 0.5[a] | 74.0 ± 8.4[a] |

[a] $n = 8$. [b] $n = 6$. [c] $n = 12$. [d] 4 mol % AAc.

**Table 2. Change in Hydrogel Volume between RT and 37 °C**

| hydrogel | swelling in PBS (days) | vol change (%)[a] |
|---|---|---|
| P(NIPAAm) | 0 | −92.0 ± 0.0 |
| P(NIPAAm) | 3 | −92.7 ± 1.2 |
| P(NIPAAm) | 6 | −96.0 ± 0.0 |
| P(NIPAAm-*co*-AAc)[b] | 0 | +5.3 ± 6.1 |
| P(NIPAAm-*co*-AAc)[b] | 3 | −17.3 ± 6.1 |
| P(NIPAAm-*co*-AAc)[b] | 6 | −41.3 ± 4.6 |

[a] $n = 3$. [b] 4 mol % AAc.

the addition of more hydrophilic monomers to a P(NIPAAm) hydrogel increases the LCST because the monomer hinders the dehydration of the polymer chains and acts to expand the collapsed structure. Specifically, hydrophilic AAc tends to increase the LCST of NIPAAm−AAc copolymers because the ionized $-COO^-$ groups (at a pH of 7.2) are sufficiently soluble to counteract the aggregation of the hydrophobic temperature-sensitive elements.[19] Also, the repulsion of the $-COO^-$ groups or the formation of hydrogen bonds between the amide groups in NIPAAm and the $-COO^-$ groups in AAc may impede the collapse induced by the NIPAAm components, increasing the LCST. In addition to an elevated LCST, the P(NIPAAm-*co*-AAc) hydrogel exhibited a broader transition than the P(NIPAAm) hydrogel, indicating decreased swelling thermosensitivity (i.e., the degree of swelling change with external temperature change[23]). Copolymer hydrogels composed of NIPAAm and a more hydrophilic monomer have demonstrated decreased swelling thermosensitivity[23,25] because the monomer prevents the formation of a compact shrunken structure. It is important to note that the LCST of the P(NIPAAm-*co*-AAc) hydrogel can be tailored by changing the amount of AAc included in the polymerization formulation.

**Water Content of the Hydrogels.** After 0, 3, or 6 days of swelling in PBS at RT, the water content of the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels was determined at RT (≈22 °C) and 37 °C. These results are shown in Table 1. At RT, the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels contained >90% water regardless of the duration of swelling in PBS. When heated to 37 °C after 0 days of swelling in PBS, the P(NIPAAm-*co*-AAc) hydrogel contained significantly more water than the P(NIPAAm) hydrogel (93.3 ± 5.4% vs 43.7 ± 7.7%; $p < 0.01$). The low water content of the P(NIPAAm) hydrogel was not unexpected since these hydrogels collapse substantially and expel a large amount of pore water when heated to temperatures above the LCST.[20−22] Interestingly, when the P(NIPAAm-*co*-AAc) hydrogel was swollen in PBS for 6 days and then heated to 37 °C, the water content dropped significantly from 93.3 ± 5.4% to 74.0 ± 8.4% ($p < 0.01$). This result may be due to interactions between the $-COO^-$ groups in AAc and the ions in PBS. The collapse resistance provided by the $-COO^-$ groups (e.g., $-COO^-$ repulsion, hydrogen bonds, etc.) may be decreased in the presence of ions. So, as the duration of swelling in PBS prior to heating increases, a comparable increase in hydrogel collapse may be observed, leading to a lower water content. In contrast, the water content of the P(NIPAAm) hydrogel increased significantly from 43.7 ± 7.7% to 67.8 ± 11.9% ($p < 0.01$) when swollen in PBS for 6 days and then heated to 37 °C.

The RT water content of the P(NIPAAm) hydrogel increased significantly from 91.5 ± 0.8% after 0 days of swelling in PBS to 95.3 ± 0.8% after 6 days of swelling in PBS ($p < 0.01$) which is consistent with both the properties of a hydrogel and previously reported data.[29,43,44] In contrast, there was no significant difference in the RT water content between the 0 and 6 day data for the P(NIPAAm-*co*-AAc) hydrogel. Furthermore, the RT water content of the P(NIPAAm-*co*-AAc) hydrogel after 6 days of swelling in PBS was significantly lower than the RT water content of the P(NIPAAm) hydrogel after 6 days of swelling in PBS ($p < 0.01$). These results were unexpected for an ionic hydrogel which typically swells to a great extent and has a high water uptake compared to that of a neutral hydrogel because the ionizable groups make the matrix more hydrophilic.[44] In the presence of PBS, Coulombic interactions between positive ions and the $-COO^-$ groups presumably disrupt many of the hydrogen bonds, interfering with the swelling of the ionic hydrogel.

**Change in Hydrogel Volume between RT and 37 °C.** The changes in hydrogel volume data are presented in Table 2. The addition of AAc to the P(NIPAAm) hydrogel significantly decreased the extent of collapse exhibited by the P(NIPAAm-*co*-AAc) matrix, as compared to the P(NIPAAm) hydrogel ($p < 0.01$). The P(NIPAAm) hydrogel collapsed >90% of its volume when heated from RT to 37 °C regardless of the duration of swelling in PBS. Such extensive collapse, commonly observed with P(NIPAAm) hydrogels,[20−22] undoubtedly contributed to the low 37 °C water content of the homopolymer NIPAAm hydrogels, as compared to the RT water content values. Prior to swelling in PBS, the P(NIPAAm-*co*-AAc) hydrogel demonstrated minimal volume change when heated from RT to 37 °C. As the hydrogel was swollen in PBS for 3 and 6 days, the volume change increased significantly ($p < 0.01$), and the hydrogel collapsed more, presumably due to interactions between the $-COO^-$ groups in AAc and the ions in PBS.

The minimal volume change exhibited by the P(NIPAAm-*co*-AAc) hydrogel prior to swelling in PBS is important from a tissue engineering standpoint. Extensive collapse of the matrix would be an undesirable characteristic of a scaffold for tissue repair. If after being injected into a defect inside the body, the scaffold collapsed to fill only a small fraction of the defect volume, the potential for successful tissue growth would be substantially decreased. In addition, extensive collapse of the matrix could squeeze the cells out of the scaffold and/or damage the cells. The +5.3 ± 6.1% change in volume demonstrated by the P(NIPAAm-*co*-AAc) hydrogel prior to swelling in PBS was statistically equivalent to a volume change of zero ($p > 0.01$). Thus, the adverse effects that could result from extensive collapse of the matrix would not be expected if the P(NIPAAm-*co*-AAc) hydrogel was injected into the body in its as-synthesized state (i.e., not swollen in PBS following synthesis). Furthermore, such a nonequilib-

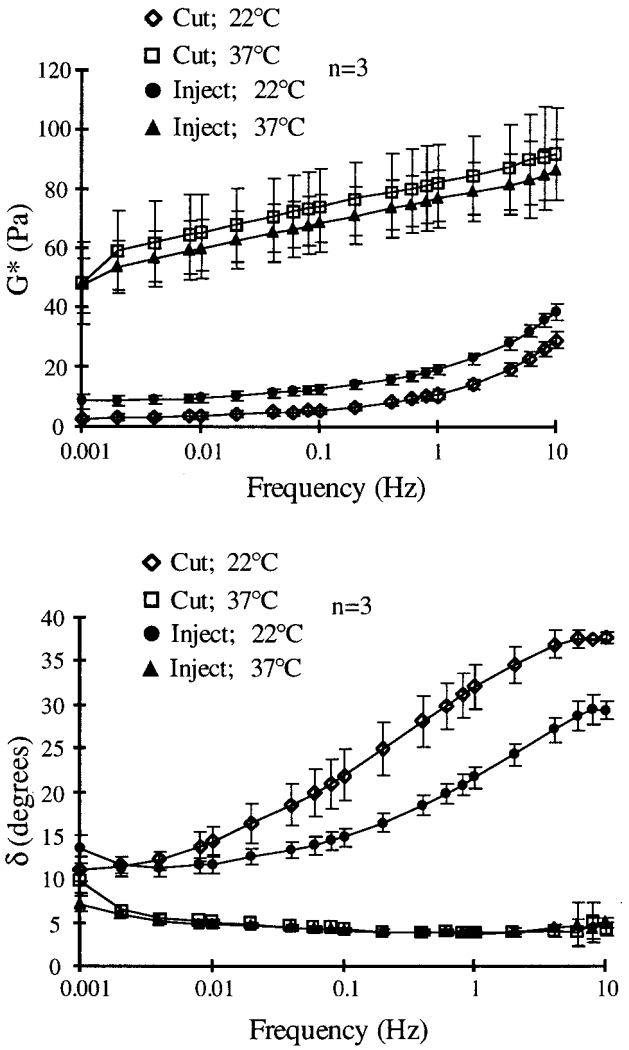



**Figure 3.** Complex modulus ($G^*$) and phase angle ($\delta$) of the P(NIPAAm-*co*-AAc) hydrogel as a function of frequency, temperature, and sample preparation after 0 days of swelling in PBS. Frequency is on a log scale. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

rium swollen P(NIPAAm-*co*-AAc) hydrogel would not be expected to swell once placed inside the body. When P(NIPAAm-*co*-AAc) hydrogels were heated in the presence of PBS without being preswollen in PBS, the volume did not significantly increase (see Table 2), and additional liquid was not absorbed (see Table 1). Prior to swelling the P(NIPAAm-*co*-AAc) hydrogels in PBS, the water content at 37 °C, which was obtained in the presence of PBS, was not significantly different from the water content at RT (93.3 ± 5.4% at 37 °C vs 92.6 ± 0.7% at RT; $p > 0.01$).

**Rheological Properties of the P(NIPAAm-*co*-AAc) Hydrogel.** Plots of the complex modulus ($G^*$) and the phase angle ($\delta$) as a function of frequency, temperature, and sample preparation are shown in Figure 3. At 22 °C, the P(NIPAAm-*co*-AAc) hydrogel rheology was characteristic of an extremely soft, cross-linked solid. As the frequency decreased, the phase angle became smaller, and the modulus approached a constant value in the range 1−10 Pa. When the temperature was increased, the gel became significantly more rigid and solidlike; note the drop in phase angle. Over the range of frequencies tested, $G^*$ increased significantly when the temperature was increased from 22 to 37 °C, regardless of the sample preparation method employed ($p < 0.01$). The water in the hydrogel did not appear to contribute substantially to the measured viscoelastic properties, since the magnitude of the loss modulus ($G''$) at 22 and 37 °C over the range of frequencies tested was at least 1 order of magnitude higher than would be predicted from viscous dissipation of water alone (data not shown). The variance in the data was due primarily to inconsistent covering of the parallel plates with the hydrogel samples.

Even though qualitatively the P(NIPAAm-*co*-AAc) hydrogel at 37 °C did not become as stiff as the P(NIPAAm) hydrogel at 37 °C, the rheological data confirm that the P(NIPAAm-*co*-AAc) hydrogel was significantly more rigid at 37 °C than at RT. From a tissue engineering perspective, this increase in stiffness represents in situ stabilization. At RT, the P(NIPAAm-*co*-AAc) hydrogel is extremely pliable and injectable through a small-diameter aperture. As the temperature is increased above the LCST to 37 °C, the hydrogel rigidity increases, and the matrix, in essence, stabilizes into a more substantial structure that may better support tissue growth.

**In Situ Viability Study.** In situ fluorescent images of bovine articular chondrocytes cultured in vitro in the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels are shown in Figure 4. The viability and shape of the cells in the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels were not qualitatively different. The chondrocytes were viable in both hydrogels for at least 28 days of in vitro culture. In addition, the chondrocytes maintained a round shape, a typical characteristic of differentiated articular chondrocytes.[45] Clumps of cells, as well as individual cells, were observed in both hydrogels. Although the cells did not completely fill the hydrogel samples, cells were seen throughout the matrices. Chondrocytes cultured in 3D alginate beads for 28 days in vitro served as the positive control for cell viability and cell shape because culture in 3D alginate beads is accepted as a method for successfully maintaining the differentiated articular chondrocyte phenotype in vitro.[46] Chondrocytes cultured on the bottom of a tissue-culture well (i.e., a 2D surface) for 28 days in vitro served as the negative control. These images are presented in Figure 5. Chondrocytes cultured in alginate beads were viable and round, and many cell clumps were observed. The chondrocytes cultured on the 2D surface were viable but appeared to have a fibroblastic morphology, an indication of chondrocyte "dedifferentiation".[47]

Since the water content of a hydrogel can represent its porosity,[29] the chondrocyte results observed in the P(NIPAAm) hydrogel were unexpected, given the low water content of the matrix (43.7 ± 7.7%; see Table 1). Generally, polymer scaffolds used in cartilage regeneration studies are >90% porous,[1,48] so a difference in chondrocyte viability was expected between the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels due to the significant difference in water content, and presumably porosity, of the matrices at 37 °C (43.7 ± 7.7% vs 93.3 ± 5.4%; see Table 1). However, there was no qualitative difference between the chondrocytes in the two hydrogels. Regardless of the low water content of the P(NIPAAm) hydrogel, the microarchitecture provided an acceptable environment that sustained chondrocyte viability inside the matrix.

**Histological Analyses.** Images of the histological data obtained from the chondrocyte-loaded P(NIPAAm)

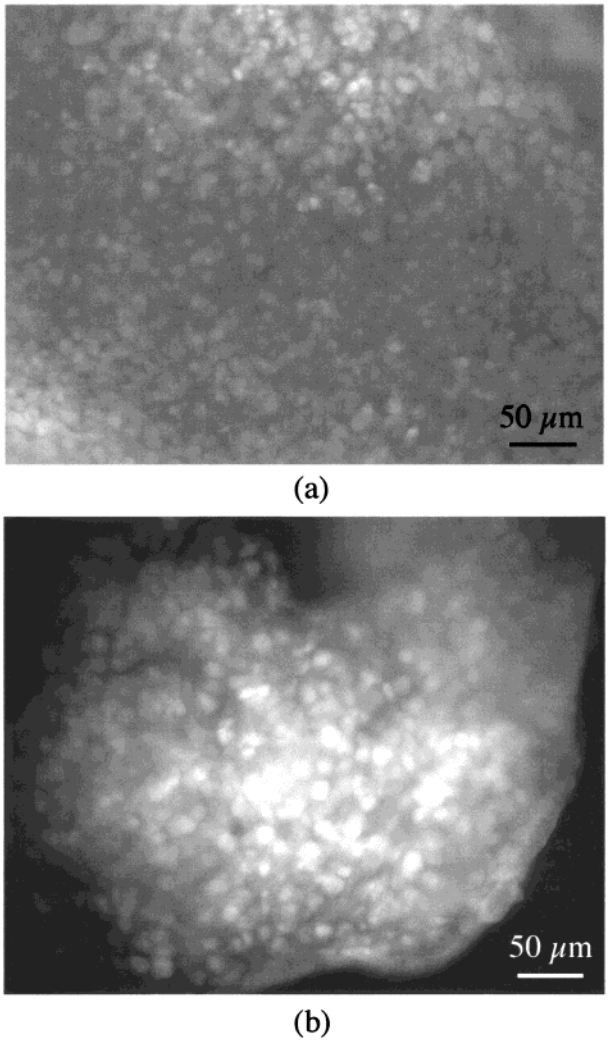



**Figure 4.** In situ fluorescent viability study of bovine articular chondrocytes in the (a) P(NIPAAm) and (b) P(NIPAAm-*co*-AAc) hydrogels after 28 days of in vitro culture. The hydrogels were not swollen in PBS prior to cell loading. The chondrocytes were seeded according to the methods described in the text and were stained with fluorescein diacetate. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

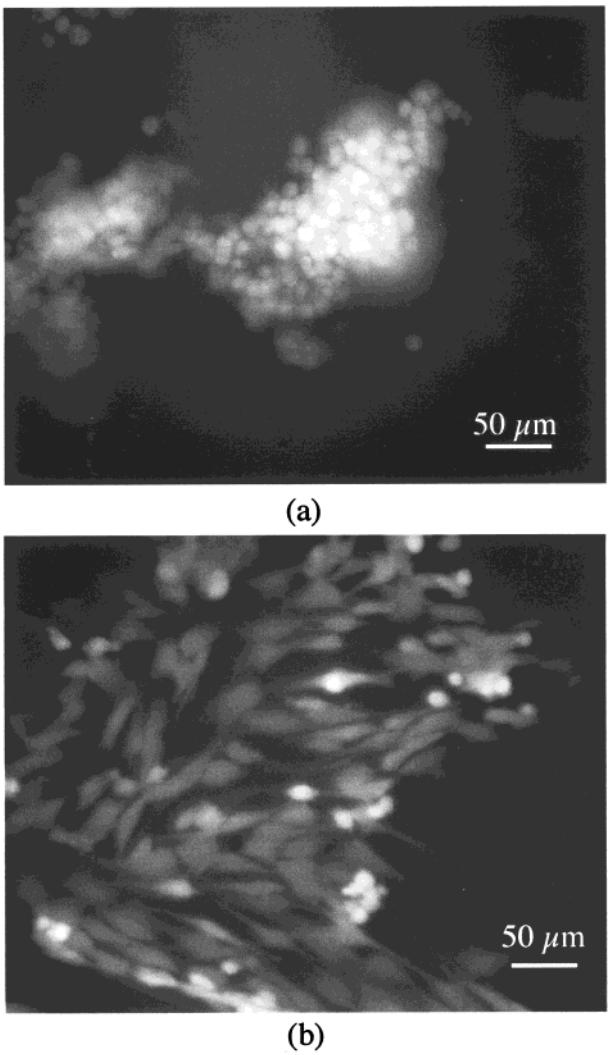



**Figure 5.** In situ fluorescent viability study of bovine articular chondrocytes (a) in alginate beads and (b) on a 2D surface after 28 days of in vitro culture. The chondrocytes were seeded according to the methods described in the text and were stained with fluorescein diacetate.

and P(NIPAAm-*co*-AAc) hydrogels are presented in Figures 6 and 7. Tissue, consisting of individual cells surrounded by an extracellular matrix (ECM), was formed in both the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels. The structure of this tissue resembles the histoarchitecture of native articular cartilage and is comparable to tissue formed in other cartilage regeneration studies.[1,3] The formation of tissue suggests that the isolated chondrocytes synthesized and secreted ECM components after being seeded into the hydrogels, a further indication that the hydrogels sustained cell viability. The cells in the tissue exhibited a round morphology and were located within lacunae. Qualitatively, the tissue formed in both hydrogels was similar; however, the cells in the P(NIPAAm) hydrogel were farther apart from each other, a characteristic that more closely resembles the cell spacing in native articular cartilage. This observation suggests that the cells in the P(NIPAAm) hydrogel synthesized and secreted more ECM components, increasing the intercellular distance. Since the P(NIPAAm) hydrogel sample was cultured in vitro almost 3 times longer than the P(NIPAAm-*co*-AAc) hydrogel sample (90 days vs 36 days), the cells in the P(NIPAAm) hydrogel presumably had more time to produce and secrete ECM components. As seen in Figure 7, the ECM of the tissue formed in both hydrogels stained positive for Alcian blue (pH = 1.0), indicating the presence of sulfated polysaccharides.[42] Alcian blue staining is generally accepted as a marker for the differentiated articular chondrocyte phenotype.[1,3]

The tissue formed in the P(NIPAAm) hydrogel stained darker than the tissue produced in the P(NIPAAm-*co*-AAc) hydrogel. The difference in color may be due to the relative amounts of sulfated polysaccharides present in the tissues. Again, the chondrocyte-seeded P(NIPAAm) hydrogel was cultured in vitro longer, so the cells presumably had more time to synthesize and secrete sulfated polysaccharides. Alternatively, the different sectioning and staining techniques used may have caused the color intensity differences observed. In future work, the amount of ECM components (e.g., type II collagen and glycosaminoglycans) will be quantified to assess the ability of the scaffolds to support cartilage tissue formation.

Large cell clusters are visible in the histological images. Since the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels are generally not considered to be degradable,

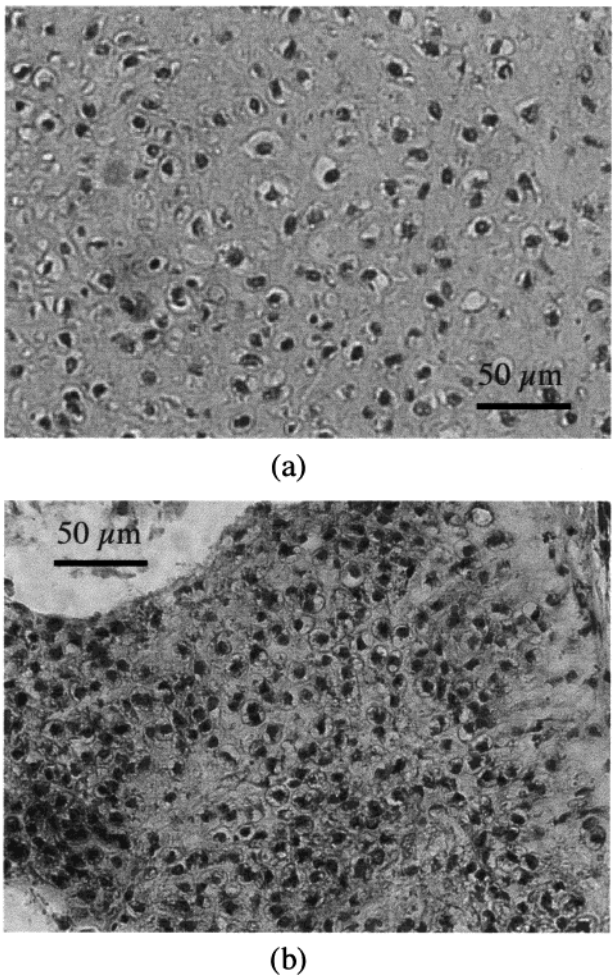



**Figure 6.** Chondrocyte-seeded (a) P(NIPAAm) and (b) P(NIPAAm-*co*-AAc) hydrogels stained with H&E. The P(NIPAAm) hydrogel section was obtained after 90 days of in vitro culture, and the P(NIPAAm-*co*-AAc) hydrogel section was obtained after 36 days. The hydrogels were not swollen in PBS prior to cell loading. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

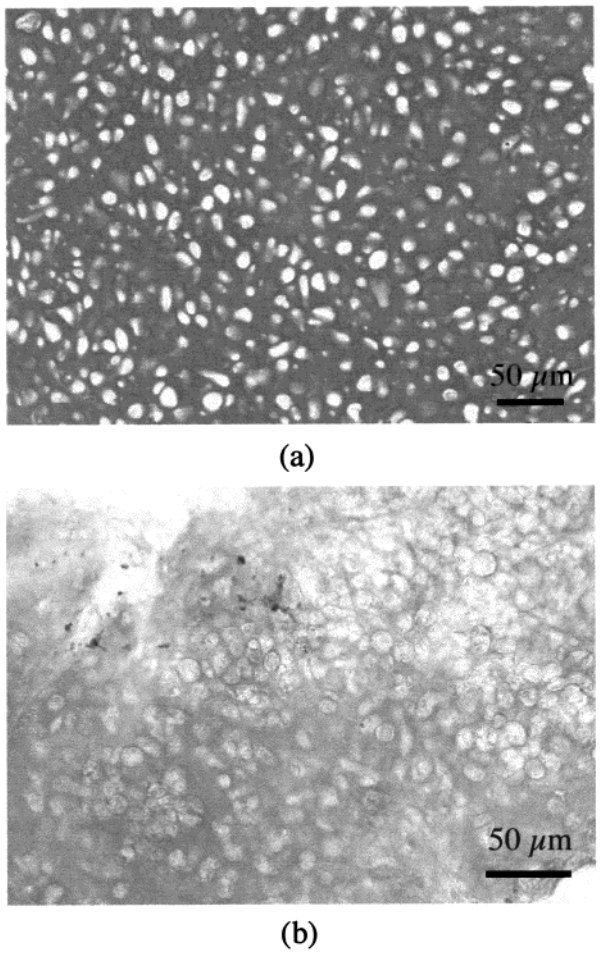



**Figure 7.** Chondrocyte-seeded (a) P(NIPAAm) and (b) P(NIPAAm-*co*-AAc) hydrogels stained with Alcian blue. The P(NIPAAm) hydrogel section was obtained after 90 days of in vitro culture, and the P(NIPAAm-*co*-AAc) hydrogel section was obtained after 36 days. The hydrogels were not swollen in PBS prior to cell loading. The P(NIPAAm-*co*-AAc) hydrogel contains 4 mol % AAc.

the question "where are the hydrogels?" seems appropriate. Large pores may conceivably be formed in the hydrogels above the LCST due to the aggregation of the NIPAAm components.[49] In addition, the groups of cells may actually deform the matrices and either push or fracture the hydrogels to create large spaces. Alternatively, amide bonds can be hydrolyzed,[50] so it is possible that the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels are actually degrading, creating large voids for cells. Although no specific tests were performed to measure cell proliferation, such large cell clusters may indicate, from a qualitative standpoint, that the chondrocytes proliferated in the hydrogels.

Degradability is an important characteristic of scaffolds for tissue engineering applications, as the risk of infection or other complications (e.g., impedance of new tissue function) can arise when a material remains in the body permanently. In preliminary studies performed in our laboratory, P(NIPAAm) hydrogels have been synthesized using hydrolytically cleavable cross-linkers composed of ethylene glycol, L-lactide, and $\epsilon$-caprolactone terpolymers. These P(NIPAAm) hydrogels degraded into a low-viscosity liquid after 13 days at 37 °C. Thus, it was possible to incorporate biodegradation into the P(NIPAAm)-based hydrogels.

The histological studies, in combination with the in situ fluorescent studies, demonstrated that the P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels sustained bovine articular chondrocyte viability in vitro and were not cytotoxic. Preliminary in vivo biocompatibility studies have been performed in our laboratory with the P(NIPAAm-*co*-AAc) hydrogels in a *nu/nu* nude (thymus-deficient) mouse model. The mice did not suffer any adverse effects from the hydrogels over a 7 month period, and there was minimal evidence of fibrous tissue encapsulation of the matrices. Although these preliminary studies are encouraging, more extensive studies are needed to better ascertain the biocompatibility and immunogenicity of the hydrogels.

## Conclusions

P(NIPAAm) and P(NIPAAm-*co*-AAc) hydrogels that support tissue formation in vitro were synthesized and characterized. Both hydrogels demonstrated a phase transition when heated to 37 °C, and both were injectable through a small-diameter aperture. The P(NIPAAm-*co*-AAc) hydrogel had a higher water content at 37 °C and demonstrated less extensive volume change when heated to 37 °C, as compared to the case of the P(NIPAAm) hydrogel. On the basis of the results from the characterization studies, the bovine articular

chondrocyte viability studies, the histological studies, and the capacity for biological functionalization, the P(NIPAAm-*co*-AAc) hydrogel has the potential to be used as an injectable polymer scaffold for tissue engineering applications.

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